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Original Article

Green tea extract decreases muscle pathology and NF-κB immunostaining in regenerating muscle fibers of *mdx* mice

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SUMMARY

Background & aims: Duchenne muscular dystrophy is a debilitating genetic disorder characterized by severe muscle wasting and early death in afflicted boys. The primary cause of this disease is mutations in the dystrophin gene resulting in massive muscle degeneration and inflammation. The purpose of this study was to determine if dystrophic muscle pathology and inflammation were decreased by pre-natal and early dietary intervention with green tea extract.
Methods: Mdx breeder mice and pups were fed diets containing 0.25% or 0.5% green tea extract and compared to untreated *mdx* and C57BL/6J mice. Serum creatine kinase was assessed as a systemic indicator of muscle damage. Quantitative histopathological and immunohistochemical techniques were used to determine muscle pathology, macrophage infiltration, and NF-κB localization.
Results: Early treatment of *mdx* mice with green tea extract significantly decreased serum creatine kinase by ~85% at age 42 days ($P \leq 0.05$). In these mice, the area of normal fiber morphology was increased by as much as ~32% ($P \leq 0.05$). The primary histopathological change was a ~21% decrease in the area of regenerating fibers ($P \leq 0.05$). NF-κB staining in regenerating muscle fibers was also significantly decreased in green tea extract-treated *mdx* mice when compared to untreated *mdx* mice ($P \leq 0.05$).
Conclusion: Early treatment with green tea extract decreases dystrophic muscle pathology potentially by regulating NF-κB activity in regenerating muscle fibers.

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disease affecting approximately one in 3500 boys.[1,2] Mutations in the dystrophin gene result in the loss of this protein from the sarcolemma of muscle fibers.[1,2] Dystrophin deficiency does not consistently produce muscle degeneration at all life stages, in all muscle phenotypes, or in all animal models.[3] In dystrophin-deficient skeletal muscle mechanical injury and proteolysis may be important factors but do not fully explain DMD pathogenesis. Mechanisms such as the immune/inflammatory response to injury appear to contribute substantially to muscle pathophysiology. Observations of activated immune cell infiltrates in dystrophic muscle suggest that the immune/inflammatory response may play a role in exacerbating the disease.[3–7] Recently, individual macrophage subpopulations have been reported to influence muscle degeneration and regeneration depending on the proportion of these cells present. M1 macrophages are cytotoxic and pro-inflammatory while M2 macrophages promote muscle regeneration.[8] Therefore, a shift in macrophage phenotype may be one mechanism that can regulate the dystrophic disease time course.[8]

Complementary and alternative medicine (CAM) approaches, including the use of botanicals, are being pursued in the amelioration of DMD. Green tea is a widely consumed beverage believed to elicit anti-oxidant and anti-inflammatory properties.[9,10] Green tea extract (GTE) is the hot water-soluble portion of unfermented *Camellia sinensis* leaves, which contains high levels of polyphenols. The polyphenols in GTE are mainly composed of the catechins: gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), and epigallocatechin gallate (EGCG).[9,11] These catechins are strong anti-oxidants that can quench reactive oxygen species (ROS) such as super oxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrile.[11] In GTE, EGCG is the most abundant polyphenol, accounting for 30–50% of total polyphenols, and is believed to provide the majority of the beneficial effects observed with green tea consumption.[12] EGCG

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may lead to decreased inflammation through its anti-oxidant properties or through other mechanisms. EGCG has been shown to have effects on several signaling pathways including blockade of NF-κB activation by inhibiting IκB kinase (IKK) activity.[10,13–18]

The anti-oxidant potential of GTE may be beneficial in treating dystrophic muscle, because oxidative stress is believed to contribute substantially to muscle pathology.[19,20] Evidence suggests that oxidative stress is involved in early disease stages and occurs before disease onset in *mdx* mice.[21] In one study, diets supplemented with GTE (0.01% or 0.05%) were provided to *mdx* breeder pairs and their pups prior to and following weaning. At age 28 days, extensor digitorum longus (EDL) muscles of GTE treated *mdx* pups had significant reductions in areas of necrosis and regeneration.[22] In a separate study, *mdx* mice treated with either GTE (0.05% or 0.25%) or EGCG (0.1%) for one to five weeks after weaning had increased anti-oxidant capacity, improved contractile properties, and decreased muscle pathology.[12] These studies indicate the GTE and EGCG may provide beneficial CAM modalities for reducing oxidative stress and decreasing dystrophic muscle pathology during early disease stages, however; the role of these polyphenols in altering muscle pathology and inflammation has not been fully characterized. This study represents a detailed time-course characterization of pathological and inflammatory markers at which pre-natal and early GTE treatment of *mdx* mice decreases muscle wasting.

2. Materials and methods

2.1. Mice

C57BL/6J and *mdx* mice were obtained from our colony. Breeding mice and offspring were maintained in a supervised laboratory animal facility in polypropylene shoebox cages. Mice were given free access to public tap water via an automatic watering system, and fed a standard pelleted diet ad libitum. Decaffeinated GTE (90DCF-T; Sunphenon) [polyphenols $>80\%$, catechins $>80\%$, (−)-epigallocatechin-3-gallate (EGCG) $>45\%$, caffeine $<1\%$], was a kind gift from Taiyo International (Minneapolis, MN). *Mdx* breeder pairs were provided standard breeding pelleted diets (7004; Harlan Teklad) with no GTE, 0.25% GTE, or 0.5% GTE. *Mdx* pups ($n=4$–6 for each diet and age group) were weaned at age 21 days and then supplied with a standard maintenance diet (2018; Harlan Teklad) containing the same percentage of GTE provided to the breeder pair from which they came. C57BL/6J breeders were fed the standard breeding diet and weaned pups ($n=3$–4 for each age group) were fed the maintenance control diet without GTE. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act.

2.2. Tissue collection

Mice of each genotype and treatment were selected at random from available cages. For tissue collection and for humane euthanasia, mice were sacrificed by carbon dioxide narcosis followed by secondary thoracotomy. Blood was collected via cardiac puncture. Tibialis anterior (TA) muscles were removed and were either flash frozen in liquid nitrogen and stored at −80 °C or fixed in 10% neutral buffered formalin until prepared for sectioning.

2.3. Serum analysis

Blood collected from mice was placed directly into Microtainer serum separator tubes (Becton Dickinson). Whole blood samples were allowed to clot for 30 min at room temperature. The tubes were then centrifuged for 2 min at 10,000*g* to separate serum. Serum creatine kinase (CK) assays were performed in the Clinical Pathology Laboratory at Virginia Tech, using an Olympus AU400 chemistry analyzer (Olympus America, Center Valley, PA).

2.4. Histopathological analysis

Formalin fixed TA muscles were prepared for light microscopy by dehydrating using increasing concentrations of ethanol and xylene as transitional solvents. Tissues were infiltrated with paraffin polymer, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) on automated staining equipment. A quantitative analysis of muscle histopathology was performed as follows. Degenerating fibers were identified as swollen eosinophilic, hyalinized, and pyknotic muscle fibers. Regenerating muscle fibers were identified by small diameter, centralized nuclei and basophilic cytoplasm. Necrotic fibers were identified as swollen muscle fibers with disrupted cell membranes, invaded by inflammatory cells. Image analysis software (Image-Pro Plus, Media Cubernetics, Inc., Silver Spring, MD) was used to analyze tissue sections for pathological markers in fields selected with battlement technique from the entire cross section of the TA muscle. A grid was superimposed over each selected field and the number of intersections that overlay pathological markers was reported as a percent of the total intersections that overlay muscle tissue.

Frozen serial sections 10 μm thick were transferred to positively charged slides for immunohistochemical staining. Sections were fixed in 2% formalin for five minutes and rinsed in PBS. Macrophages were identified by staining for anti-F4/80 (1:500; Serotec). After fixation, sections to be stained for NF-κB were permeabilized in 1% Triton X-100, rinsed in PBS, and covered with anti-NF-κB p65 specific for phosphorylated Serine 536 (1:100; Abcam). Sections were incubated with primary antibody overnight at 4 °C, rinsed in PBS, and immunodetection was performed using an anti-rat or anti-rabbit IgG labeled with horseradish peroxidase–diaminobenzidine system (R&D Systems). Additional sections were also incubated without primary antibody to ensure the presence of minimal background staining. The sections were then mounted and cover slipped for evaluation. The presence of macrophages and NF-κB staining was quantified using image analysis software (Image-Pro Plus, Media Cubernetics, Inc., Silver Spring, MD).

2.5. Statistics

Data were analyzed in a completely randomized design. Differences in muscle histopathology and serum CK were analyzed to determine the significance of the main effects and interactions. For the histopathology time course, the model was analyzed as a 2×5 factorial arrangement for genotype (C57BL/6J and *mdx*) versus age (14, 21, 28, 35 and 42 days). For histopathology and serum CK in GTE treatment studies, the model was analyzed as a 2×3 factorial arrangement for age (28 and 42 days) versus treatment (0%, 0.25%, 0.5% GTE). To determine the significance of the model, two-way analysis of variance (ANOVA) was performed using the general linear model. When the model was significant, the analysis was followed by Fisher's Least Significant Difference multiple comparisons method (JMP 6.0.2 software. SAS Institute Inc., Cary, NC). Predetermined comparisons between GTE treated *mdx* mice and control *mdx* mice were made using Dunnett's multiple comparisons method. Differences in macrophage

infiltration and NF-kB staining between GTE treatment groups were analyzed with one-way ANOVA. When significant differences were detected, Tukey's Honestly Significant Difference post hoc test was used to determine differences between means. Differences for all analyses were considered significant at $P \leq 0.05$ and data were presented as the mean ± standard error.

3. Results

3.1. Microscopic muscle lesions in mdx mice

Muscle histopathology was quantified for *mdx* mice during the initial disease onset stages to further characterize and determine time points that represent early disease progression (Fig. 1). Systematically selected fields from TA muscles for both C57BL/6J and *mdx* mice were quantified (Fig. 2). *Mdx* TA muscles had normal fiber morphology at age 14 days, but by age 21 days the percent area of analyzed fields with normal fiber morphology was significantly decreased compared to C57BL/6J and was further decreased at age 42 days ($P \leq 0.05$). Degenerating muscle fibers peaked at age 21 days and represented 3.5% of the *mdx* TA area, but declined to lower levels thereafter. Immune cell infiltration of the muscle tissue and necrotic fibers were seen at age 21 days and peaked at age 28 days. At age 28 days immune cell infiltration and necrotic fibers accounted for 3.6% and 1% of abnormal morphology, respectively. Regenerating muscle fibers were the most abundant histopathological feature accounting for 28% to 61% of the TA area between ages 28 and 42 days ($P \leq 0.05$). Ages 28 and 42 days were selected as the primary time points for determining the effects of GTE on muscle pathology and inflammation because they represent critical time points in the disease process of the *mdx* mouse.

3.2. GTE treatments in mdx mice

Body mass was recorded for treated *mdx* mice and compared to control *mdx* mice to determine how GTE supplementation affected overall health. GTE supplementation did not result in a significant change in body mass for either treatment when compared to untreated *mdx* mice at either age (Average body mass in grams: 28 day: *mdx* = 13.4, *mdx* 0.25% GTE = 15.9, *mdx* 0.5% GTE = 13.8; 42 day: *mdx* = 21.6, *mdx* 0.25% GTE = 21.8, *mdx* 0.5% GTE = 22.6).

3.3. Systemic indicator of muscle damage

Serum CK activity was not significantly decreased in GTE treated mice at age 28 days (Fig. 3). However, at age 42 days there was a significant decrease (~83–85%) in 0.25% (1345.5 U/L) and 0.5% (1194.3 U/L) GTE treated *mdx* mice when compared to untreated *mdx* (7896.5 U/L) ($P \leq 0.05$). There was no significant difference in CK activity for age 28 days when compared to age 42 days. CK activity for GTE treated *mdx* mice was still considerably elevated when compared to C57BL/6J mice (86.8 U/L).

3.4. Muscle lesions in GTE treated mdx mice

Histopathological features of GTE treated and untreated *mdx* mice were not significantly different at age 28 days. By age 42 days, 38% of the TA area in untreated *mdx* mice had normal fiber morphology, while 50% and 48% normal morphology was observed for 0.25% and 0.5% GTE treated *mdx* mice, respectively (Figs. 4 and 5). This corresponds to a significant improvement of 32% (0.25% GTE) and 26% (0.5% GTE) in normal fiber morphology for GTE treated *mdx* mice ($P \leq 0.05$). At age 42 days regenerating fibers were the primary histopathological feature that accounted for 61% of the TA area in untreated *mdx* mice, but only 48% in 0.25%, and 50% in 0.5% GTE treated *mdx* mice. This is a significant decrease of 21% (0.25% GTE) and 18% (0.5% GTE) in regenerating fibers when compared to untreated *mdx* mice ($P \leq 0.05$). There were no significant changes in the percent area of immune cell infiltration, necrosis, and degeneration. These markers do not appear to contribute substantially to the increase in normal fiber morphology observed in GTE treated *mdx* mice.

Macrophage infiltration is a major histopathological feature of the dystrophic disease processes (Fig. 6). Infiltrating macrophages are found throughout *mdx* TA muscles with dense foci near necrotic, degenerating and regenerating muscle fibers. GTE is thought to reduce inflammation; however, GTE treatment at 0.25 or 0.5% did not significantly reduce macrophage infiltration in *mdx* mice age 42 days.

3.5. NF-κB in necrotic and regenerating fibers

NF-κB is a transcription factor that regulates the expression of many inflammatory genes.[23] An antibody specific for the phosphorylated p65 (p-p65) subunit of NF-κB was used to determine if GTE had an effect on activated NF-κB immunostaining (p-NF-κB) (Fig. 7). Activated NF-κB is normally sequestered to nuclei; however, in DMD patients necrotic muscle fibers have been reported with cytoplasmic p-NF-kB immunostaining.[24] In TA muscles from *mdx* mice age 42 days, fibers with cytoplasmic staining were also observed. To confirm that cytoplasmic staining was specific to these fibers, negative control sections were stained without primary antibody. All negative control sections showed minimal background staining, no staining of nuclei, and no fibers with cytoplasmic staining were observed (data not shown). In tissues probed with anti-p-NF-κB, fibers with cytoplasmic staining were typically invaded or surrounded by inflammatory cells that also stained positive for p-NF-κB. The presence of inflammatory cells in and around fibers with cytoplasmic NF-κB staining indicates these muscle fibers were undergoing the process of necrosis. There was no difference in the number of fibers with

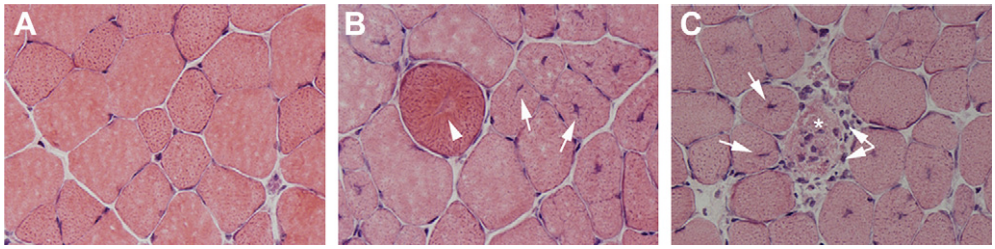

Fig. 1. Histopathological features of TA muscles from C57BL/6J and *mdx* mice age 28 days. (A) Normal muscle morphology was seen in C57BL/6J muscle sections. (B and C) Regenerating fibers (arrow), degenerating fibers (arrowhead), necrotic fibers (asterisk) and immune cell infiltration (double arrow) were apparent in *mdx* muscle sections. 400× H&E.

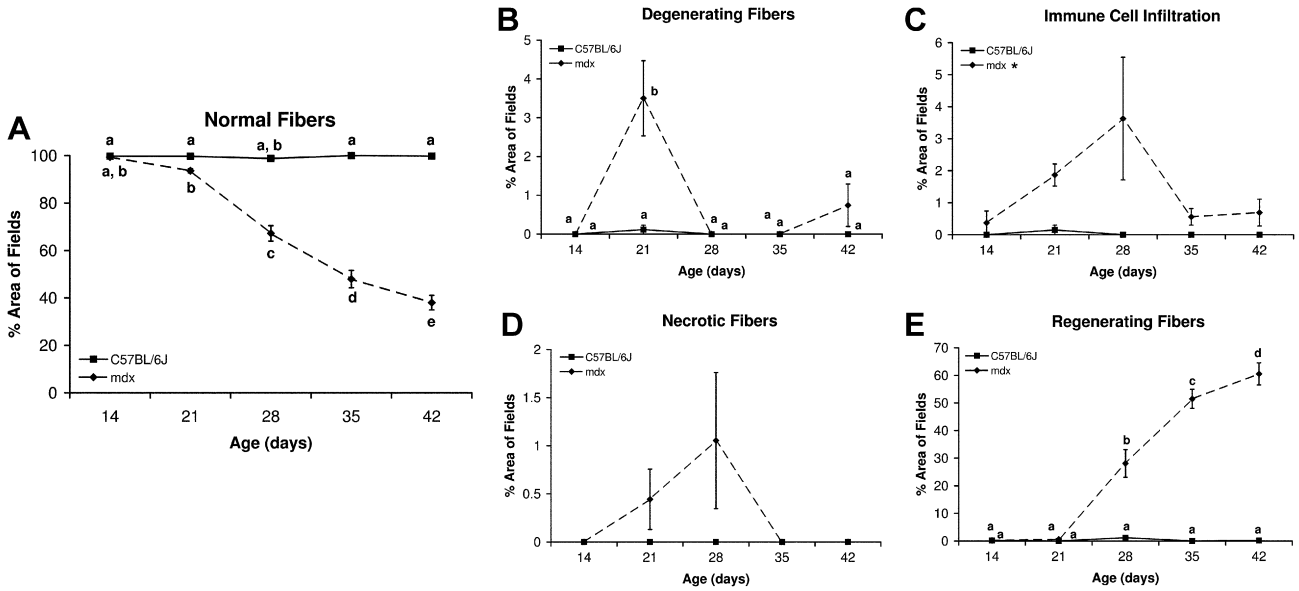

Fig. 2. Histopathology time course for C57BL/6J and *mdx* TA muscles. (A) Fiber morphology appears normal in *mdx* mice at age 14 days, but by 21 days (B) degenerating fibers and (C) immune cell infiltration were evident, followed by the formation of (D) necrotic fibers and (E) regenerating fibers by age 28 days. Mean values not connected by the same letter were significantly different ($P \leq 0.05$). *Mean *mdx* values greater than C57BL/6J ($P \leq 0.05$).

cytoplasmic NF-κB staining for GTE treated and untreated *mdx* TA muscles at age 42 days (~5 fibers/mm^2 of muscle tissue). As expected, the number of *mdx* fibers with cytoplasmic NF-κB staining was elevated when compared to C57BL/6J control (0.1/mm^2 of muscle tissue). After being phosphorylated, NF-κB enters the nucleus to regulate gene expression. NF-κB has been implicated in muscle fiber regeneration and may contribute to the dystrophic disease process.[25] Nuclei in C57BL/6J mice had little or no immunostaining. Both GTE treated and untreated mice had strong immunostaining in peripheral and centralized nuclei. Strong staining in regenerating fibers was observed for 44% of centralized nuclei in untreated *mdx*, while only 29% and 30% were observed in 0.25% and 0.5% GTE treated *mdx*, respectively. This was a significant decrease of 34% (0.25% GTE) and 31% (0.5% GTE) for GTE treated *mdx* mice when compared to *untreated mdx* mice ($P \leq 0.05$).

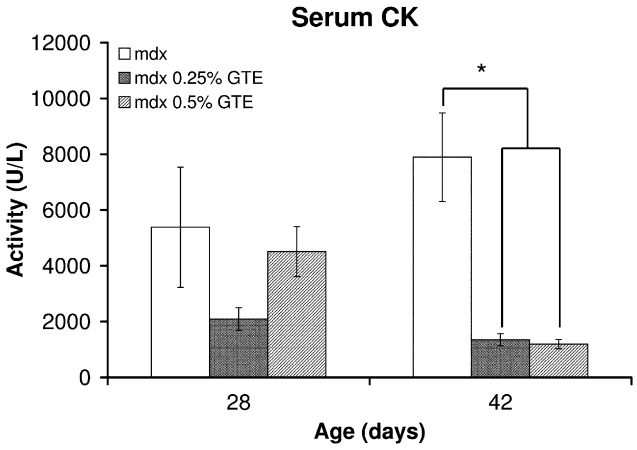

Fig. 3. Serum CK for *mdx* and GTE treated *mdx* mice age 28 and 42 days. *Mdx* mice treated with 0.25% or 0.5% GTE had significantly reduced serum CK values when compared to untreated *mdx* mice at age 42 days. *Mean untreated *mdx* values were significantly different compared to treated *mdx* mice ($P \leq 0.05$).

4. Discussion

The objective of this study was to characterize and identify distinct features of dystrophic muscle pathology during the early disease time course and determine what features of dystrophic muscle pathology were reduced by pre-natal and early dietary intervention with GTE. Recent studies have relied on the percent area of centrally nucleated fibers and the area of necrotic muscle tissue to assess changes in muscle pathology and often combine these features to determine overall affected muscle tissue.[7,12,22,26–32] However, these studies have not discriminated the percent area

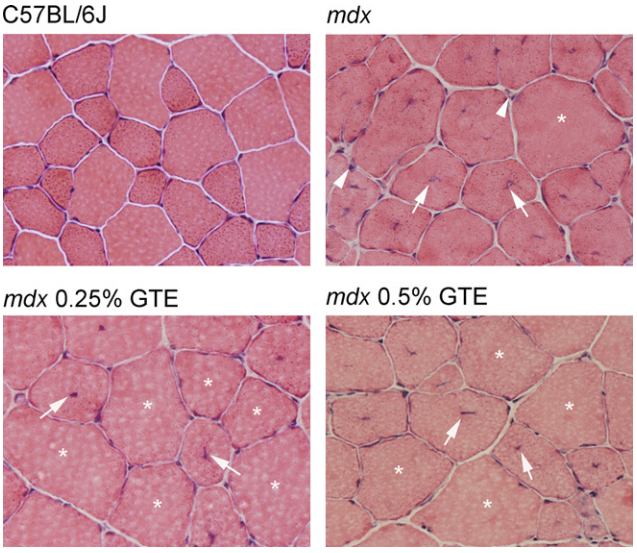

Fig. 4. Histopathology of TA muscles for C57BL/6J, *mdx* and GTE treated *mdx* mice age 42 days. C57BL/6J muscle sections depicting normal fiber morphology for this age. Untreated *mdx* sections showing a morphologically normal fiber (asterisks), regenerating fibers (arrow), and immune cell infiltration (arrowhead). Treated *mdx* sections from 0.25% GTE and 0.5% GTE had altered muscle pathology with an increase in morphologically normal fibers (asterisks) and fewer regenerating fibers (arrow). 400× H&E.

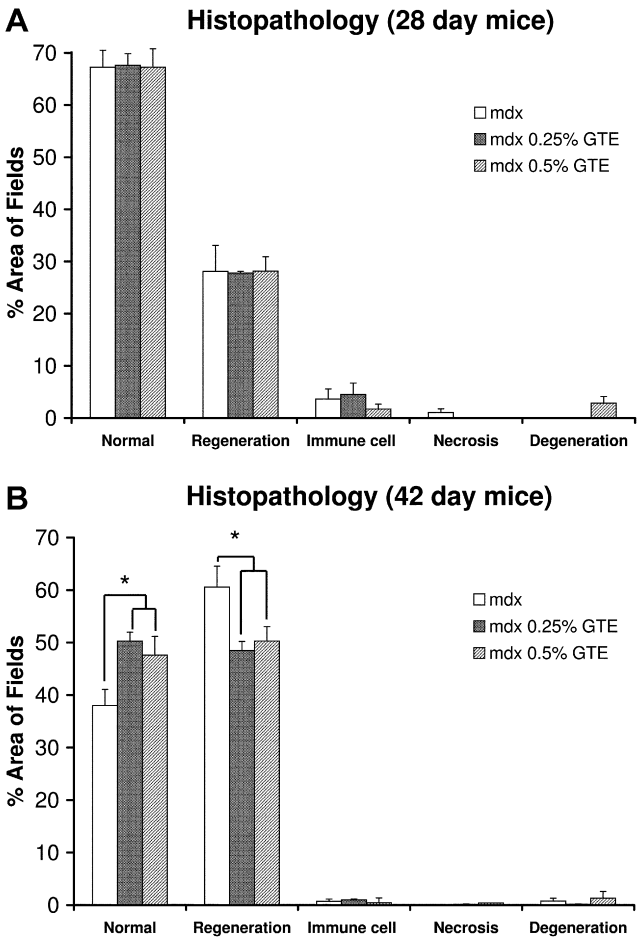

Fig. 5. Quantification of histopathology for *mdx* and GTE treated *mdx* TA muscles at ages 28 and 42 days. (A) Although necrotic fibers and fiber degeneration/regeneration were apparent in *mdx* TA muscles by age 28 days, there was no difference in histopathology in GTE treated *mdx* mice compared to untreated *mdx* mice at age 28 days, (B) By age 42 days there was a significant increase in normal fiber morphology and a decrease in regenerating fibers in GTE treated *mdx* mice. *Mean untreated *mdx* values were significantly different compared to treated *mdx* mice ($P \leq 0.05$).

of the individual fibers undergoing the histopathologically identifiable processes of degeneration, necrosis or regeneration. Therefore, it is important to note that whole muscle tissue necrosis/degeneration values that are often reported are not the same as the percent area of fiber necrosis or fiber degeneration reported here. One limitation to this approach is that the total area of tissue necrosis may not be fully realized. To compensate for this limitation, areas of immune cell infiltration where there was ongoing tissue necrosis outside of intact fibers were also measured as a separate histopathological marker and quantification of the number of infiltrating macrophages was also reported.

The time course of histopathology shows that the percent area of normal fiber morphology is the same for C57BL/6J and *mdx* TA muscles at age 14 days, but by age 21 days there is a significant decrease in the percent area of normal fibers for *mdx*. This time point represents the onset of disease for the *mdx* mouse. Normal fiber morphology at all other ages analyzed was significantly decreased for the *mdx* mouse. The percent area of muscle fiber necrosis, degeneration, and immune cell infiltration represent important features of the disease process between ages 21 and 28 days, but overall account for only small percentages of the abnormal fiber morphology (~4.5–6%). The percent area of regenerating fibers represents the largest portion of abnormal fiber morphology at ages 28 (28%) to 42 (61%) days. The large percent area of regenerating fibers compared to other histopathological features indicates that the regenerative process is a substantial contributor to dystrophic muscle pathology. Studies detailing the process of muscle regeneration have shown that in damaged muscle tissue, fibers are capable of returning to normal fiber morphology; however, many regenerated fibers retain centrally located nuclei indefinitely.[33–36] The persistence of centrally located nuclei is believed to signify that abnormal processes are occurring in these cells that prevent the nuclei from returning to the periphery of the cell.[33–36] From this initial analysis, it was determined that for the GTE treatments muscle pathology should be examined at ages 28 and 42 days because these time points represent the early degenerative and regenerative disease stages.

Serum CK is a systemic indicator of muscle pathology. CK is normally restricted to the cytoplasm of muscle fibers, but when the fibers are damaged it is released into the serum. Serum CK was not different for GTE treated *mdx* mice compared to untreated *mdx* at age 28 days, but at age 42 days there was a significant improvement in GTE treated *mdx* mice. Similarly, there was no difference in

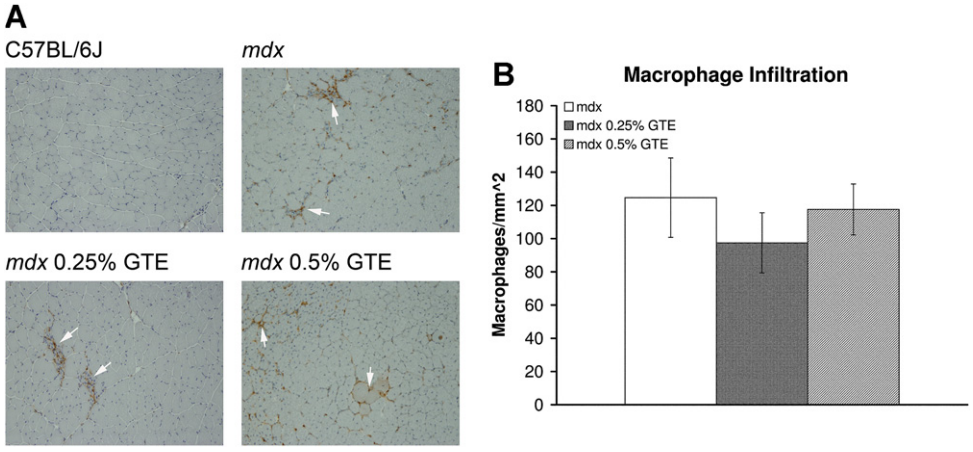

Fig. 6. Macrophage infiltration (F4/80) of TA muscles from mice age 42 days. (A) C57BL/6J muscle sections had very few infiltrating macrophages. Untreated *mdx* sections had macrophage infiltration (arrow) throughout with dense foci of macrophages. Treated *mdx* sections from 0.25% GTE and 0.5% GTE had similar macrophage infiltration (arrow) compared to untreated *mdx*. 100×. (B) Quantification of macrophages revealed there was no difference in the number of infiltrating macrophages for GTE treated and untreated *mdx* mice.

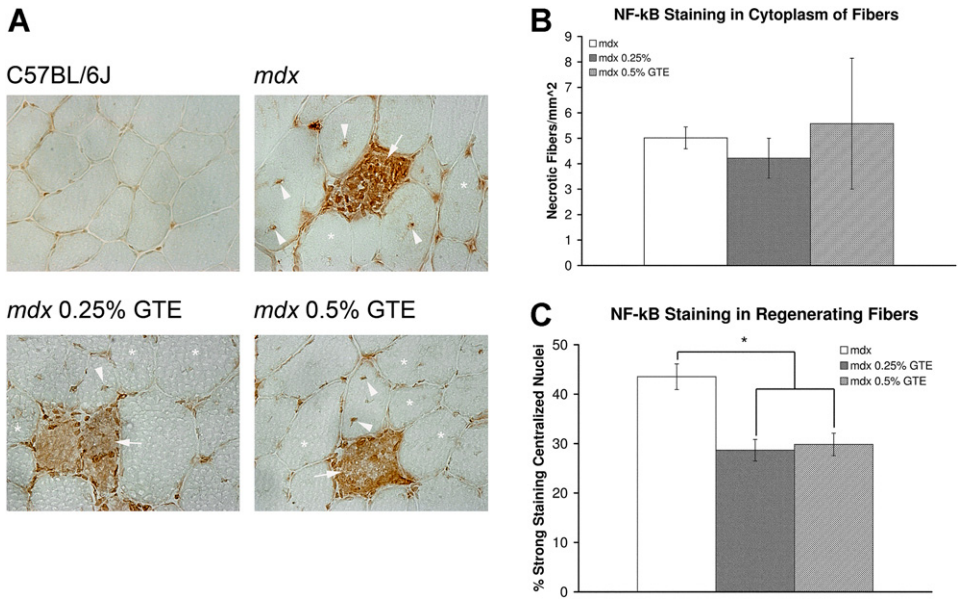

Fig. 7. Anti-p-NF-κB (p-p65) staining of TA muscles from mice age 42 days. (A) C57BL/6J muscle sections had no cytoplasmic staining and weak nuclear staining. Untreated *mdx* sections had staining in peripheral nuclei and the cytoplasm of necrotic fibers infiltrated by inflammatory cells (arrow). Regenerating fibers had centralized nuclei with both strong (arrowhead) and weak (asterisk) immunoreactivity. Treated *mdx* sections from 0.25% GTE and 0.5% GTE had the same number of infiltrated necrotic fibers with cytoplasmic staining (arrow), but had a decrease in the percent of regenerating fibers with strong immunoreactivity of centralized nuclei compared to untreated *mdx*. 400×. (B) Quantification of p-NF-κB (p-p65) staining revealed there was no difference in the number of fibers with cytoplasmic staining for GTE treated and untreated *mdx* mice. Cytoplasmic NF-κB staining in muscle fibers was indicative of necrotic fibers. (C) In regenerating fibers GTE treated *mdx* mice had a significant decrease in strong centralized nuclear staining. *Mean untreated *mdx* values were significantly different compared to GTE treated *mdx* mice ($P \leq 0.05$).

normal fiber morphology for GTE treated and untreated *mdx* mice at age 28 days; however at age 42 days there was a significant improvement for GTE treated *mdx* mice. There was up to a 32% increase in normal fiber morphology for these mice. The improvement in normal fiber morphology was largely accounted for by up to a ~21% decrease in regenerating fibers. Other histopathological features were unchanged by GTE treatments. Collectively this data indicates that overall muscle pathology was decreased in GTE treated *mdx* mice.

To further assess how GTE treatments modulate inflammation; macrophages and NF-κB immunostaining were quantified. There was no difference in the number of macrophages infiltrating the TA muscles in GTE treated or untreated *mdx* mice. Although GTE is believed to have anti-inflammatory properties, it does not appear to decrease the number of infiltrating macrophages in *mdx* mice at age 42 days. However, a recent study reported that muscle from *mdx* mice contains two subpopulations of macrophages, M1 and M2.[8] M1 macrophages are thought to be cytotoxic and pro-inflammatory while M2 macrophages inhibit the cytotoxicity of M1 macrophages and promote muscle regeneration. In *mdx* mice there was a shift in the macrophage phenotype to the regenerative subset between age 4 weeks and 12 weeks.[8] Although there was no change in the overall number of infiltrating macrophages in GTE treated *mdx* mice age 42 days, one possibility is that GTE induces a shift in the phenotype of infiltrating macrophages. If GTE treatments did contribute to a shift in macrophage phenotype from M1 to M2 without affecting the total numbers of macrophages, it could result in an improved regenerative process. However, further studies are needed to address this possibility.

A component of GTE, EGCG, has been reported to inhibit IKK activity which results in inhibition of NF-κB.[10,13–15] Acharyya et al.[25] recently reported *mdx* muscles lacking IKKβ, a subunit of the IKK complex, have an increase in regenerative capacity when compared to control *mdx*. They also found that *mdx* muscles with macrophages lacking IKKβ had decreased muscle pathology and a decrease in the expression of pro-inflammatory makers.[25] Their data suggest a mechanism through which NF-kB signaling in macrophages and regenerating muscle fibers promotes degeneration and represses regeneration.[25] Several studies have reported an increase in the number of regenerating fibers after treatments that reduce or block NF-κB activity while others have reported a decrease in regenerating fibers.[25,28,29,37,38] These differing results may stem from the way in which the NF-κB pathway is altered by treatment compounds and the methods used to administer these compounds (i.e. age of treatment initiation and termination, route of administration). Our findings indicate that pre-natal and early dietary GTE treatment reduces the amount of regenerating fibers potentially through alterations in NF-κB signaling. Past studies have also shown a decrease in muscle necrosis when NF-κB activity is inhibited.[25,28,29] Our results indicate that the amount of fiber necrosis is unchanged by GTE treatment. Additionally, the number of fibers with cytoplasmic NF-κB immunostaining was unchanged by the GTE treatment. The presence of inflammatory cells infiltrating fibers with cytoplasmic NF-κB staining signifies these muscle fibers were undergoing necrosis. These findings suggest that while muscle degeneration and necrosis initiates the process of muscle wasting, impaired muscle regeneration plays a key role in the progression of muscular dystrophy.[25,28,29]

GTE has been shown in several studies to decrease serum CK levels and muscle pathology while increasing force output, and endurance capacity in *mdx* mice.[12,22,30,39] The anti-oxidant properties of GTE are believed to be the primary element responsible for these improvements, other signaling pathways may also be responsible. Previous reports have shown that GTE treatment can result in a significant decrease in muscle pathology,[12,22] which is supported by this study. However, past studies did not discriminate the different pathological markers that were reported here, including the finding that the primary change was in the percent area of regenerating fibers. In this study and previously we reported that serum CK was decreased with pre-natal and early GTE

treatment. Serum CK is an indicator of the extent of muscle damage and a significant decrease of this marker indicates that GTE is having an important impact on dystrophic muscle pathology. Additionally, no other studies have attempted to determine the effect of dietary GTE on inflammation. Although no significant change in infiltrating macrophages was observed, a decrease in NF-κB immunostaining in regenerating fibers was observed. NF-κB is a known regulator of inflammatory genes and may play an important role in the dystrophic disease process and the recruitment of inflammatory cells. GTE role in inflammation may prove to be an important component of the decreased *mdx* muscle pathology.

CAM approaches, including the use of anti-inflammatory botanicals in general and GTE in particular, may lead to the amelioration of DMD and provide important insight into disease processes. CAM interventions can be used in conjunction with conventional therapies in a cost-effective manner to improve disease prognosis. GTE treatments of *mdx* mice resulted in increased normal fiber morphology, decreased regenerating fibers, and decreased NF-κB staining in regenerating fibers. The detailed histopathological analysis revealed that changes in the percent area of degenerating fibers, necrotic fibers, and immune cell infiltration were small in comparison to the change in regenerating fibers. This data indicates that GTE decreases NF-κB activity in regenerating fibers which may result in changes in the regenerative process leading to an increase in normal fiber morphology. Detailed muscle histopathology for pre-natal and early GTE treated *mdx* mice has not been previously reported and reveals that GTE may be an additional treatment option to decrease early muscle pathology for this incurable disease. In conclusion, GTE decreases muscle pathology potentially by suppressing NF-κB activity in regenerating fibers of *mdx* mice. While GTE did not decrease overall immune cell or macrophage infiltration, additional studies are needed to examine the role of GTE in modulating the shift of macrophages towards and M2 phenotype and thereby ameliorate the dystrophic disease process by favoring anti-inflammatory responses.

Conflict of interest

The authors have no conflict of interest.

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